

INOCULATION EXPERIMENTS WITH *BACILLUS FUSIFORMIS* ISOLATED FROM TROPICAL ULCER WITH OBSERVATIONS ON THE BACILLUS.

By E. C. SMITH, M.D.

(From the Medical Research Institute, Lagos, West Africa.)

(With Plate I, containing Figs. 1-10.)

In a previous communication (Smith, 1931) mention has been made of the growth of fusiform bacilli in so-called symbiosis with the spirochaetes commonly associated with typical tropical ulcer. Subcultures, subsequently made on various kinds of solid media and grown both aerobically and anaerobically, proved negative. As a result of further experiments, however, it has been found possible to isolate these bacilli and maintain cultures of them through numerous passages. The anaerobic plate method, as described by Krumwiede and Pratt (1913), was finally adopted as it gave the most consistent results. Fragments of tissue from the surface of an ulcer, previously cleaned carefully with warm sterile water, were placed in flasks each containing 65 c.c. of liquid serum agar (sheep serum 25 c.c., agar 50 c.c.), the agar having been previously melted and cooled to 56° C. before adding the serum. A fragment teased out in a drop of sterile water was examined by dark ground illumination to make certain that motile fusiform bacilli were present in the material and, in addition, smears were examined in order to gain some estimate of the amount of contamination present. If little or no contamination was noted, several minute fragments were placed in each flask and, after shaking, the contents were poured, the amount (65 c.c.) being found sufficient for two plates. These were incubated at 37° C. The *fusiformis* colonies were rarely recognisable before the third day. When a plate is held in front of a black background and viewed by transmitted light with a $\times 10$ lens the colonies appear as minute translucent or slightly cloudy irregularly shaped growths lying in the substance of the medium. Colonies of *Bacillus pyocyaneus*, streptococci and diphtheroids were almost always present, all giving colonies recognisable by the second day. At the fourth or fifth day the fusiform colonies usually showed, scattered through their substance, a greyish mottling or stippling giving them a characteristic appearance. In some of them, a tendency to form blunt outgrowths was noticed, the resultant colonies resembling a miniature hat with a highly pointed crown. Thread-like prolongations, as noted by Krumwiede and Pratt (1913), were not observed. Though the stippled and hat-shaped colonies referred to looked entirely different from the ordinary ones, no marked dis-

similarity could be made out in their component bacilli when examined by dark ground illumination and in stained smears. Grouping or clustering of the fusiform bacilli was a marked feature of the plate cultures, and it was noticed that growth was most pronounced when contamination with a *mesentericus*-like organism occurred; suggesting that this bacillus or the products of its growth were beneficial to that of *B. fusiformis*. Numerous experiments were made later, filtrates of broth cultures of this bacillus and also killed cultures being added to the media in which the fusiform bacilli were grown, but no uniform results were obtained.

MAINTENANCE OF GROWTH.

Owing to the translucent or opalescent appearance of the colonies, some difficulty was experienced when subculturing. Varney (1927) recommends the examination of surface growths with a dissecting microscope, using reflected light and a tilted stage. This method was adopted, but without the tilted stage. The upper plate of the petri-dish being first removed from the surface of the subjacent media by a sudden twisting movement, a suspected colony was then brought into focus and "fished" or cut out of the media by means of a finely sharpened platinum wire and placed on a thin slide. A suitable cover-slip was pressed firmly on top and the preparation examined by dark ground illumination. It was found more convenient to mount the minute agar particle containing the colony without the addition of any diluent. Having determined the presence of fusiform bacilli, similar colonies can be picked out into a large drop of broth in a petri-dish, then emulsified with a platinum spud and distributed into further plates or tubes as required.

ATTEMPTS AT PRODUCING SURFACE GROWTHS FROM KRUMWIEDE PLATE CULTURES.

Emulsions in broth of fusiform colonies obtained from plates in the manner previously described were spread on serum (of horse and sheep) agar slopes and plates and incubated anaerobically for periods up to ten days but with negative results. In addition, plates made with the media described by Gins (1930) and Varney (1927) and after the method of Fortner (1928) were tried and also gave negative results.

CULTURES IN SEMI-SOLID MEDIA.

Growth was obtained in semi-solid serum agar (horse serum 25 parts, agar 10 parts, broth 50 parts) under anaerobic conditions. In a paper on communal activity in bacteria by Churchman and Kahn (1921) the relation between the volume of medium and the inoculated organism is discussed and it was decided to try the effect of cultivation in small amounts of media. Dwarf test-tubes ($3\frac{1}{2} \times \frac{3}{8}$ in.) were employed, anaerobic conditions being maintained by the use of a modified form of Buchner tube (Fig. 10) in which complete anaerobiosis, as shown by clearing of the methylene blue indicator

tube put up with each set of cultures, could be maintained for at least 7 days. Dwarf tubes (containing *ca.* 2 c.c. of medium) when incubated under these conditions showed a definite enrichment of the cultures as compared with those grown under similar conditions in large tubes. Sanarelli (1927) has described the marked effect produced by *B. mesentericus*, or filtrates of this organism, upon the growth of fusiform bacilli. A strain of *B. mesentericus*, kindly supplied by the Lister Institute, was added to cultures of *B. fusiformis* in dwarf tubes and fourteen subcultures of this mixed growth were made at 7-day intervals. In the earlier subcultures a reinforcement of growth took place, the fusiform bacilli appearing as dense clumps or sheaves, intermingled with the coarser *B. mesentericus* (Fig. 4). Gradually, however, the former were outgrown until finally only scanty fusiform bacilli could be found. The addition of filtrates of broth cultures of *B. mesentericus* to cultures of fusiform bacilli did not result in any apparent amelioration of growth. The pure cultures were entirely free from odour, this being in agreement with the observations of Sanarelli (1927) and Gins (1930) as against those of Tunncliff (1906), Ellermann (1904) and others who described such cultures as having a bad odour. When using the dwarf tubes and growing anaerobically, growth was maintained for seven subcultures, made at intervals of 8 to 10 days. The cultures of *B. fusiformis* + *B. mesentericus* were subcultured fourteen times, subcultures being made every 5 days. After this period (about 70 days) the cultures gradually weakened and became overgrown by *B. mesentericus* as already stated.

MOTILITY.

In all the colonies examined on the third or fourth day by the dark ground method, motile individuals were present. In some colonies they were relatively few, and these showed only a sluggish penetrative motion. In other colonies the whole field swarmed with actively motile forms. The type of motility was characteristic, being actively progressive combined with a curious side to side oscillation. This was accentuated when chains of the bacilli were encountered; in these the foremost member of the group showed a marked side to side motion, the remainder being drawn passively along in its wake. These chains of bacilli remained motile in old cultures even when movement was not noted in the surrounding fusiform organisms. In fresh cultures individuals endowed with unusually rapid movement crossed the field from time to time, cleaving their way amongst their more staid companions. In general, the more elongate and typically spindle-shaped forms were the more actively motile. Sanarelli (1927) has given an excellent description of these organisms, comparing the chains of bacilli to a series of minute boats. He regards such forms as being composed of a single member, twisted upon its axial filament. The shorter stubbed forms might easily be passed over for large motile bacilli with bluntly pointed ends were it not for their characteristic beaded appearance when stained. Individual bacilli with centrally situated globular swellings and long mycelial forms were frequently noticed under the dark

ground. It was found that the fusiform bacilli remained actively motile for 15 days at room temperature (25–27° C.), after which time the motility gradually diminished until at 20 days only scanty, feebly motile, bacilli were seen.

STAINED SMEARS.

For rapid results, staining with freshly prepared Leishman was excellent, but for general use Giemsa was found to be the most suitable. Smears were fixed in methyl alcohol and stained 48 hours in a 1 per cent. dilution of the stain in distilled water; they were then treated with concentrated tannic acid solution until pink, passed rapidly through graded acetone-xylol mixtures and mounted in cedar-wood oil. The differentiation so obtained was excellent. When young cultures (4–5 days) were studied, the organisms from different colonies showed great variation (Figs. 1–3), but those in any one colony were fairly uniform in shape and staining reactions. Beading or granulation was the predominant feature, the granules being situated either at definite intervals in the body of the organism or at its extremities. In some, the granules assumed a bi-polar band-like form. These forms correspond more or less to the types described by Knorr (1930). In older colonies (7–15 days) pleomorphism was marked, long filiform beaded forms being intermingled with short stumpy varieties, the latter frequently showing only one central granule. The chain-like forms previously referred to were more frequent in the older cultures. Curious atypical forms were also seen in such old cultures though they were occasionally observed in the younger growths. The more common form was that in which a central globular or elliptical swelling was present in the organism. In some a bending of the bacillus occurred at this swelling, giving it a peculiar joint-like appearance. These swellings were non-acid fast and did not react to spore stains. Exactly similar forms could be found in smears made direct from ulcers (Figs. 5, 6). Prolonged staining by Giemsa brought out chromatin-like structures in some of these atypical forms, suggestive of mitosis. Long thick mycelial forms with blunt ends occurred in most of the older colonies and usually appeared in the form of one or more coarse spirals. In occasional straight forms of this type an inner thread-like filament (Fig. 9), resembling a free spirochaete, could be demonstrated by intensive Giemsa staining. Tunncliffe (1923) describes similar appearances and apparently regards these forms as developing spirochaetes. In this connection it must be noted that motile spirochaetes were never found associated with the fusiform bacilli in pure culture, but in some instances numerous non-motile organisms, strongly resembling spirochaetes, were found associated with the young third day cultures of fusiform bacilli.

EXPERIMENTAL INOCULATIONS ON VOLUNTEERS.

The results of inoculations made on volunteers I–VIII are recorded in Tables I and II.

In addition to these eight experiments, two more were made using twelve well-formed 5-day colonies of fusiform bacilli picked out from a serum agar culture (made with material from a typical ulcer) and emulsified in 1 c.c. of broth in a Griffith's tube. A loopful of this emulsion was examined by the dark ground and smears were made and stained by Leishman and Gram; actively motile fusiform bacilli, showing typical beading, were found. The emulsion was then inoculated intracutaneously in equal amounts into two Europeans (into the deltoid region in one and the inner aspect of the ankle in the other). In both, an angry red halo with central induration occurred within 24 hours. This gradually increased up to 48 hours when regression took place.

The foregoing experiments show that pure cultures of a strain of fusiform bacilli, grown in solid and semi-solid media, when inoculated intracutaneously into volunteers did not produce lesions resembling tropical ulcer, although there was evidence of considerable toxic action. When experimentally inoculated into the skin the bacilli remained alive as shown by the case of Volunteer I, in whom motile fusiform bacilli were found in the exudate 14 days after inoculation. Further, the addition of *B. pyocyaneus* to this inoculum did not cause typical ulceration. No mutation into spirochaetes was observed either in cultures or experimental lesions. The results in five cases show that the inoculation of material obtained from experimental lesions into man and animals (guinea-pigs and monkey) does not reproduce the condition.

SUMMARY.

The isolation of fusiform bacilli from typical tropical ulcer is described and an account is given of the motility and staining properties of the organisms cultivated.

Experimental inoculations, both in man and animals, are recorded in tabular form and the results of the investigation discussed.

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Table I. *Inoculations with pure cultures of Bacillus fusiformis.*

A first subculture (grown in serum agar) was used. Volunteers I, II and III were each inoculated in the deltoid region with 1 c.c. of an agar growth emulsified in broth. Controls received serum agar alone.

Volunteers	1st day	8th day	11th day	12th day	14th day
I	Indurated painful area at site of inoculation. Rise in temperature to 99° F. Complained of headache	Indurated area increased to 1 inch in diam. Soft centre. Pus aspirated. Negative for organisms in smears, dark ground and agar culture. Temperature normal. Headache still present	Inflamed boil-like lesion. Brownish-red purulent discharge. Gram-stained smears show slender fusiform bacilli. Dark ground examination shows motile fusiforms. No spirochaetes. Some of the discharge emulsified in broth and inoculated, in amounts of ½ c.c., into volunteers IV and V. Krumwiede plate cultures also made. (These positive for fusiforms in pure culture after 2 days)	"Boil" still present. Findings in discharge as before. Some emulsified in broth and ½ c.c. inoculated into volunteer VI. One c.c. also inoculated into two guinea-pigs and one <i>Macacus rhesus</i> . (Results in these animals negative)	"Boil" regressing. Findings in discharge as before. Negative for other organisms in smears and cultures
II	Negative	Slight induration and pain at site of inoculation	Subsiding	Normal	
III	Indurated painful area	Indurated area with soft centre. Pus aspirated, negative for organisms	Subsiding	Normal	

IV-VI The inoculations on volunteers IV-VI, made with the material indicated above under 11th and 12th day, did not produce any marked result. In two, a bleb formed after 24 hours which contained a turbid fluid—negative for organisms. Autoinoculation of this fluid produced no result.

Table II. *Inoculations with pure cultures of B. fusiformis in one site and B. fusiformis + B. pyocyaneus in another site.*

Volunteers	Upper area	1st day (24 hours)	2nd day	3rd day	4th day	5th day	11th day
VII	Upper area	—	Vesiculation present	Deep pus formation	Purulent bleb, 1 cm. in diam. Smears show scanty fusiforms. No spirochaetes	Depressed bleb. Scanty fusiforms. No spirochaetes	Drying up smears negative
	Lower area	Vesiculation present	Vesicle burst. Seropurulent discharge. <i>B. fusiformis</i> scanty, <i>B. pyocyaneus</i> + +	Bleb formed. Seropurulent discharge	Same condition as 3rd day	Small central slough and surrounding ulcerated area. Not typical of tropical ulcer. Smears:— <i>B. fusiformis</i> , <i>B. pyocyaneus</i> + +	Drying up smears negative
VIII	Upper area	—	Negative	—	—	—	—
	Lower area	Marked induration	Vesiculation around a central bleb	Vesicles fused into large bleb	Condition as on 3rd day	Bleb discharging. Fusiforms scanty. No spirochaetes. <i>B. pyocyaneus</i> + + +	Drying up

Note. In all experimental inoculations, the inoculum was examined under the dark ground for the presence of actively motile fusiforms. The controls, apart from slight redness and induration after 24 hours, remained negative.

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EXPLANATION OF PLATE I.

Figs. 1-9. *Bacillus fusiformis*, × 1000.

- Fig. 1. 5-day culture. Slender forms with well-marked beading.
- Fig. 2. 5-day culture. Short bluntly pointed forms and dividing forms.
- Fig. 3. 5-day culture. "Train" of bacilli in centre.
- Fig. 4. 5-day culture. Fusiforms growing in presence of *B. mesentericus*.
- Figs. 5 and 6. Smears from tropical ulcer, showing pleomorphic and atypical fusiforms.
- Fig. 7. 8-day culture. Atypical forms, suggestive of mytosis.
- Fig. 8. 15-day culture showing "joint-like" forms.
- Fig. 9. 10-day culture showing spiral structure within a spirochaete. (Photomicrographs by Mr F. W. Randall.)
- Fig. 10. Modified Buchner tube used for anaerobic cultivation. 1. Large glass test-tube.—2. Dwarf tubes.—3. Glass specimen tube.—4. Fibre disc with opening to allow glass tube to pass through. (See text, p. 96.)

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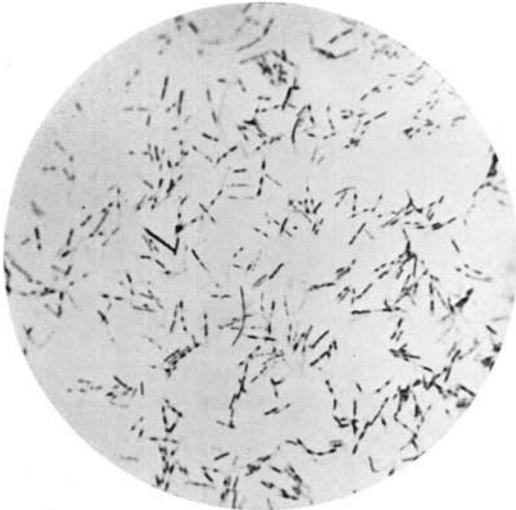


Fig. 1.



Fig. 2.



Fig. 3.

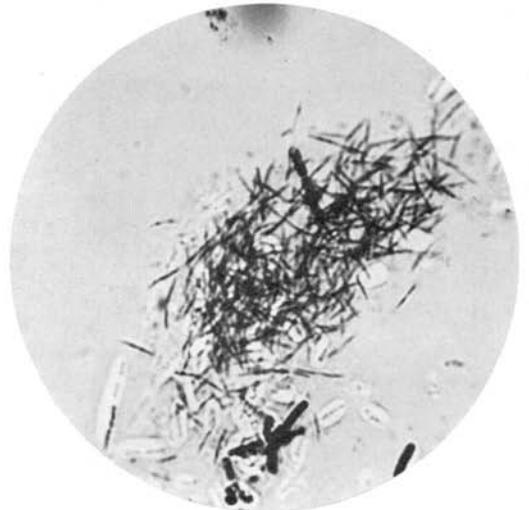


Fig. 4.

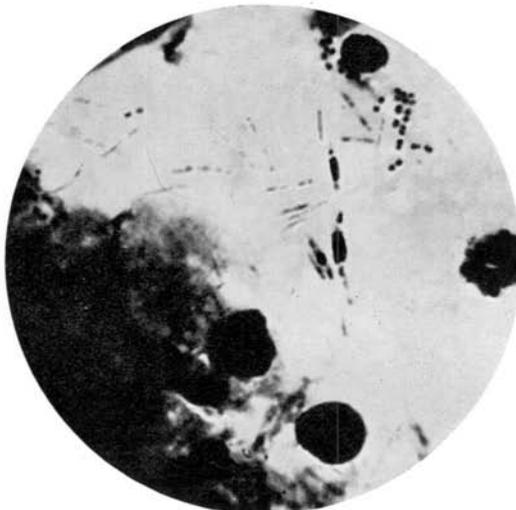


Fig. 5.

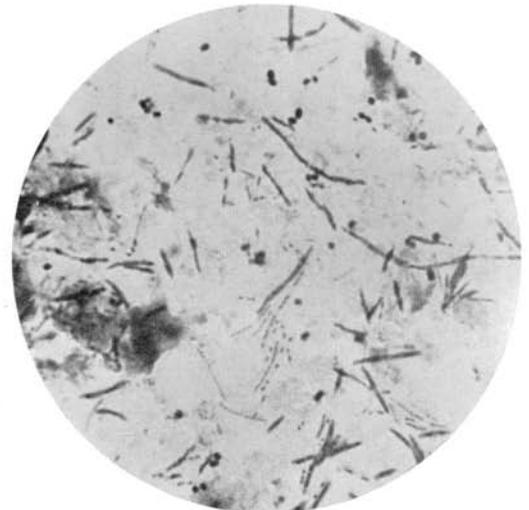


Fig. 6.

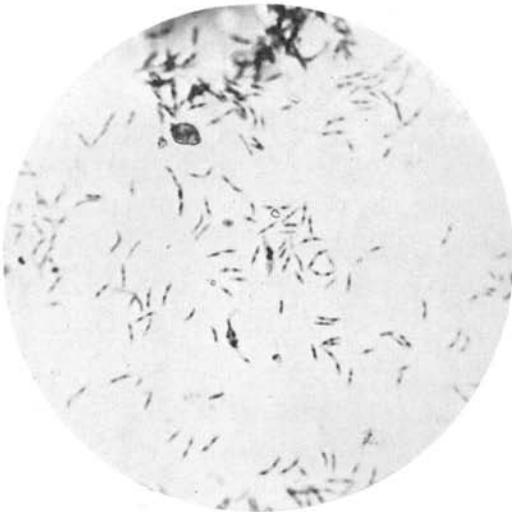


Fig. 7.

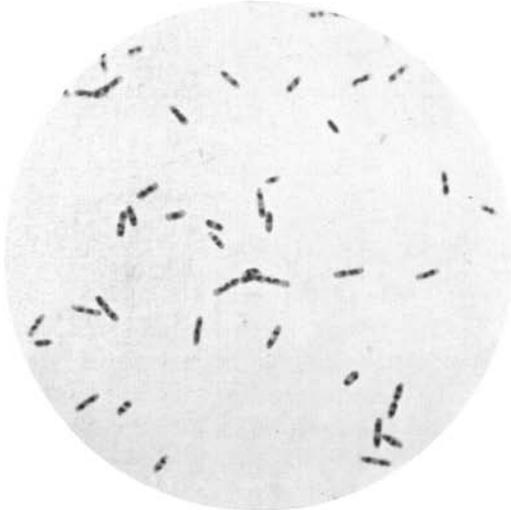


Fig. 8.



Fig. 9.

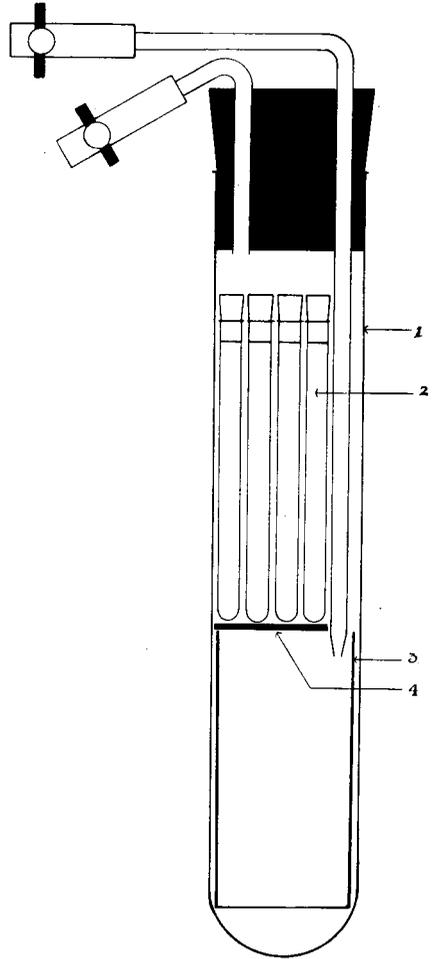


Fig. 10.